The Regulation of Smooth Muscle-Specific Gene Expression by the Methyltransferase, PRDM6

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Abstract

Smooth muscle cell (SMC) differentiation is controlled by a variety of co-factors to activate SMC-specific gene expression. We identified the methyltransferase, PRDM6, as a myocardin factor binding factor. Interestingly, PRDM6 expression in mouse and humans is highly SMC-selective. Genome wide association studies (GWAS) identified a locus in the PRDM6 3rd intron that is associated with blood pressure (BP) and intracranial aneurysm. Since this locus is also associated with differences in PRDM6 expression in human aortic samples, we hypothesize that this locus affects the activity of a regulatory element in this region. Based upon several genome wide data sets, we identified a region in the PRDM6 3rd intron that drove Luciferase expression in a SMC-specific fashion. Furthermore, to identify the regions that regulate PRDM6 expression in SMC, we mutated transcription factor binding sequences in a highly conserved region of the PRDM6 3rd intron (Int3.1cons) and found that these mutations decrease PRDM6 activity in SMC. Through the use of Chromatin immunoprecipitation (ChIP) assays, we identified that the Int3.1cons region significantly bound to three known SMC differentiation transcription factors: SRF, RBPJ, and TEAD1. We also show preliminary data that suggests that these transcription factors interact to coregulate PRDM6 expression. Altogether, this will help us to further understand PRDM6 function and effects as they relate to cardiovascular diseases.

Background and Significance

Differentiated smooth muscle cells (SMC) provide structural support to the vasculature and control blood pressure (BP) and blood flow. Defects in the maintenance of SMC differentiation contribute to atherosclerosis and hypertension. Since SMCs are defined by the expression of the functional genes, known as differentiation markers (i.e. SM myosin heavy chain (MHC), SM α-actin, etc.), further identifying the transcriptional regulation of these genes will increase our understanding of vascular development and vascular disease.

Several factors are known to contribute to the regulation of SMC differentiation. For example, serum response factor (SRF) regulates SMC-specific transcription by binding to the conserved CArG ($CC(A/T)_6GG$) cis elements in their promoters [1, 2]. However, SRF is not a smooth muscle specific transcription factor. Yet, the family of Myocardin factors has been identified as non-DNA binding regulators of SMC differentiation. Expression of Myocardin is not only heart and SMC-specific, but it was also shown to induce SMC differentiation marker gene expression in non-SMC, and upon deletion of Myocardin in the mouse model, lethality was observed in part due to the lack of SMC differentiation of the dorsal aorta[17,18]. Furthermore, Myocardin physically interacts with the non-specific SRF, forming homodimers, to co-activate SMC-specific genes expression [3, 4].

Another highly conserved regulatory pathway that drives vascular differentiation is the Notch signaling pathway. Notch is a single-pass transmembrane receptor that interacts with the Jagged and Delta-like ligand families on adjacent cells, which causes the cleavage of Notch intracellular domain (NICD) by γ-secretase and the subsequent translocation of NICD to the nucleus where it binds to Recombination Signal Binding Protein For Immunoglobulin Kappa J (RBPJ), the main DNA binding protein of the Notch signaling pathway in SMC[6]. Namely, Notch

signaling has been shown to be necessary for arteriovenous differentiation[6]. However, the effects of Notch signaling are controversial, in regards to the activation or inhibition of SMC differentiation gene markers, which could be explained by the effects being in a time-dependent fashion. We have previously shown that the transcription factor, RBPJ, represses the expression of SMC-specific genes[5]. Yet notably, NICD binds RBPJ and reverses the RBPJ-dependent repression of SM-markers[6], and thus can also activate SM-specific gene expression through recruitment of MAML[5]. With the knockout of Notch in mice, arterial vascular SMC-specific markers (smoothelin and SM22) decrease as well, providing more evidence of up regulation of SM-specific gene expression[6]. Interestingly, through the analysis of ChIP-seq data, RBPJ binding sites are consistently identified within the same open chromatin binding loci as SRF, and the Mack lab has shown that these important transcription factors interact to co-regulate gene expression essential for proper SMC function[5].

Chromatin structure is also a major contributor to the regulation of gene expression. Histone acetylation and methylation, depending on the amino acid, can lead to open chromatin regions that increase the accessibility of genes to transcriptional machinery. DNAse sequencing is a technique used to identify regions across the genome that are open chromatin regions by enzymatically cleaving the accessible regions of euchromatin. Further, DNAse sequencing not only provides data about genes that are accessible, but also open regions that regulate transcription of specific genes. The Mack Lab used this DNAse hypersensitivity model to better characterize the changes in frequently euchromatic regulatory regions that were SMC-specific, when compared to other cell types (see Figure 1). Many labs have shown that histone and DNA modifications that regulate chromatin structure also contribute to SMC-specific gene expression[9,10], although many critical questions remain, especially in regard to the cell-type- and

gene-specificity of regulatory pathways and epigenetics, as well as about how chromatin modifying enzymes are recruited to the SMC gene promoters.

We recently identified the putative methyltransferase, PRDM6, as a myocardin related factor-A (MRTF-A) binding partner using a co-IP/mass spec-based approach, and this result was confirmed by co-immunoprecipitation and Far Western analysis. Because PRDI-BFI/RIZ domain proteins (PRDMs) share homology with the SET (Suvar/Enhancer of zest/Trithorax) family, it is thought that PRDMs control cell fate decisions by functioning as histone methyltransferases and/or by interacting with positive and negative chromatin remodeling enzymes[11]. Interestingly, PRDM6 expression in mouse and humans is highly SMC-selective [12,13], but the effects of PRDM6 on SMC gene expression are highly controversial. We have previously shown that siRNA-mediated knockdown of PRDM6 in rat aortic SMC decreased SMC differentiation marker gene expression suggesting that PRDM6 and MRTF-A act as co-regulators. However, Davis et al have reported that the knockdown of PRDM6 stimulates the expression of Myocardin and SMC differentiation markers[12]. Yet, to further support the importance of PRDM6 in SMC, a familial human genetic study described PRDM6 coding mutations that were associated with patent ductus arteriosis [14], a syndrome caused by defects in the contraction and remodeling of this embryonic vessel. In addition, separate GWAS have identified a locus in PRDM's third intron that was associated with BP regulation and intracranial aneurysm[15,16]. As observed in Genotype-Tissue Expression data, this locus was associated to differences in PRDM6 expression in human aortae ($p=2.3e^{-5}$) strongly suggesting that the control of PRDM6 expression is critical for normal SMC function. Because this locus is within the PRDM6 third intron we hypothesized that it effects PRDM6 expression by altering the activity of a regulatory element in this region.

The goals of my project were to identify the regions of the third intron that regulate PRDM6 activity, identify key transcriptional elements that help regulate PRDM6 expression, analyze the effects that PRDM6 knockdown had on SM markers and test whether PRDM6 expression splice variants had an effect on SM markers.

Methods

Regulatory region cloning- Several regulatory regions within the PRDM6's BP-associated locus were PCR amplified from human aortic smooth muscle genomic DNA and then cloned into the pGL3 basic vector (Promega). These regions include the Int3.1, Int3.2, Int3Plus, Int3.1cons, Int3.1cons mutations, and TSS pieces. The Int3.1 region was cloned using a XhoI restriction enzyme site to cut the pGL3 vector and insert, before ligating them together. The plasmids were then transformed into bacteria using a heat shock protocol. In brief, the plasmids were added to the DH5α competent bugs at a 1:4 ratio and were placed on ice for 10 minutes. The tubes were then placed in a 42°C water bath for 1.5 minutes and then placed on ice again for 2 minutes, followed by 4 volumes of LB media added to the tubes. The tubes were then placed in a 37°C shaker for 30 minutes and then plated on agar petri plates. After the plates sat overnight in a 37°C incubator, colonies were picked and grown up in 3mL LB media. Following the exponential proliferation in LB media, the bacterial bugs were lysed using a Crack-Lysis Buffer and the plasmid DNA samples were ran on an agarose electrophoresis gel to confirm that the cloned intron piece was successfully inserted into the plasmid. The positive results from the crack screen were then prepped with a Mini prep kit and subjected to restriction digest to confirm the direction of the inserted piece.

The Int3.2 piece and Int3Plus piece were cloned using the same methods, however they were inserted into the pGL3 vector using a PCR infusion cloning reaction.

Luciferase assay and activity- Human and mouse aortic SMC and mouse endothelial cells were seeded in 24-well culture plates. Triplicate wells were transfected with 50 ng of promoter-reporter DNA. After 48 hours of incubation at 37°C, luciferase assays were performed using the Steady-Glo Luciferase Kit (Promega). Raw luciferase values were normalized to the activity of the promoterless pGL3 from parallel experiments.

Knockdowns and Western Blots- Dharmafect siRNA were designed and then transfected into a 6-well plate containing the HuAo smooth muscle cell line. Three of the wells contained the siRNA construct, while the other 3 wells were transfected with a control siRNA that targets the GFP molecule. After a 48-hour incubation at 37°C, the cells were lysed and scraped for proteins. Two PRDM6 knockdowns wells and two control wells were separated and prepped with RNeasy kits to isolate the RNA. Finally, the RNA preps were then used for a cDNA synthesis and subsequent reverse transcriptase PCR, where fluorescence was measured based on the amount of double-stranded cDNA, and the PRDM6 was compared to the GAPDH control.

The other PRDM6 knockdown well and control well were used to conduct a BCA assay to quantify the amount of protein present in each well. The proteins were then ran using an SDS-PAGE Western blot, transferred the gel to a membrane, and probed for PRDM6 expression levels and SM marker expression using specific primary and rabbit and mouse secondary antibodies for the proteins. The film was then developed with multiple exposures to be able to indicate levels of SM and PRDM6 proteins present, compared to the GFP target controls.

Overexpression with Luciferase Assay- Human and mouse aortic SMC were seeded in 24-well culture plates. Triplicate wells were transfected with 25 ng of promoter-reporter DNA

and 25 ng of TF-secondary messenger expression vector. After 48 hours of incubation at 37°C, luciferase assays were performed using the Steady-Glo Luciferase Kit (Promega). Raw luciferase values were normalized to the activity of the promoterless pGL3 vector + Flag (or Myc) EV from parallel experiments.

Chromatin Immunoprecipitation Assay- HuBrSMC were seeded in 150mm culture plates. Once at a confluency of 80-90%, cells were fixed and DNA was cross-linked by adding formaldehyde to split media at a final concentration of 0.7% and rocking for 5 minutes at RT. Then, 1M Glycine was added, the plates were washed twice with cold 1X PBS and then frozen in -80F. Plates were thawed on ice and scraped with 3mL/plate of Cell Lysis Buffer (w/ 1:100 Phosphatase and Protease inhibitors). Cell lysates were combined from plates and centrifuged at 2300g for 5 minutes at 4°C, then resuspended in 1X PBS and recentrifuged. The nuclear pellet was then completely resuspended in Nuclear Lysis Buffer (250uL/million cells). The lysate was then sonicated (9 cycles, 10-2 second pulses/cycle, @80 Amps). The protein concentration was obtained by BCA assay and adjusted to 0.8ug/uL. Antibodies were then fixed to magnetic beads and the protein was Immunoprecipitated as per Dynabeads (Invitrogen) protocol. The protein and bound DNA were then eluted from the beads and the protein was denatured using 4ul of 5M NaCl and 2ul of Proteinase K, overnight at 65°C in a water bath. Then, the DNA fragments are isolated using the ZYMO ChIP DNA Clean-Up Kit and PCR reactions are conducted using the below conditions.

Total volume=15uL	PCR Conditions
7.5uL- Red Taq 2X Premix	94°C- 1 minute
4.5uL- dH_2O	94°C- 30 seconds
1uL- 5' Primer	61°C- 30 seconds
1uL- 3' Primer	72°C- 30 seconds
1uL- ChIP sample	72°C- 4 minutes 10°C- indefinite

Statistics

Statistical significance was assessed in data using unpaired T-tests or ratio-paired T-tests. Statistical significance was determined at a 95% confidence interval, if the p-value<0.05 (*). Statistically more significant values were recorded as the p-value<0.005(**), and the p-value<0.0005(***). Statistics are mentioned in supplemental with the format (T-test type, T, p-value, df).

Results

Identifying regulatory regions that drive PRDM6 expression in SMC. We used the following criteria to prioritize our examination of potential regulatory regions within the PRDM6 gene: 1) DNAse hypersensitivity determinations in human aortic SMC (HuAoSMC) with particular attention paid to SMC-selective DHS regions; 2) positioning of regions in relation to expression quantitative trait (eQTL) and blood pressure loci that link genetic variations to PRDM6 expression and diseases; 3) the binding of SRF and RBPJ (known regulators of SMC differentiation) as measured by ChIP seq; 4) mammalian sequence conservation in non-coding regions which typically associate with regulatory activity, and 5) Chromatin modifications associated with active promoter/enhancer elements (H3K4 methylation and H3K27). My overall approach was to PCR amplify potentially interesting regions from genomic DNA prepared from our HuAoSMC cultures, clone these fragments into the appropriate luciferase vector (into pGL3 basic vector to assess promoter function or pGL3-SV40 promoter vector to assess enhancer function), and then transfect the promoter/enhancer-Luciferase plasmids into various cell types to assess cell-type specific activity.

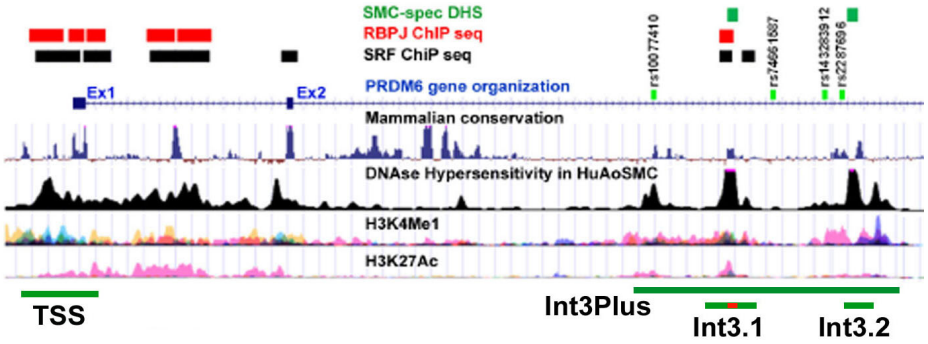


Figure 1. Schematic of the proximal region of the PRDM6 gene that summarizes our DNAse hypersensitivity data, sequence conservation, SRF and RBPJ binding, chromatin modifications, location of variants within the BP-associated LD block, and the regulatory regions tested (green lines) (Int3.1cons- red line within Int3.1 green line). Modified from Bai & Mangum et al. J Clin Invest. 2017 Feb 1;127(2):670-680.

Based on our criteria, we focused on the following regions (see Figure 1); **Transcription start site (TSS)** - this region contains the proximal promoter region, exhibits strong DNAse hypersensitivity, H3K4 methylation, and H3K27 acetylation, and contains multiple SRF and RBPJ binding sites. **Intron 3 region 1 (Int3.1)** - this region contains strong DHS region that was SMC-specific, SRF and RBPJ binding sites, strong H3K4 methylation and H3K27 acetylation, and a highly conserved 300 bp region. **Intron 3 region 2 (Int3.2)** - this region encompasses a SMC-specific DHS region and exhibits high levels of H3K4 methylation and H3K27 acetylation. Notably, the general region Intron 3 region that we are examining contains many single nucleotide polymorphisms (SNPs) in high linkage disequilibrium (LD) that are associated with hypertension, intracranial aneurysm, and differences in PRDM6 mRNA levels.

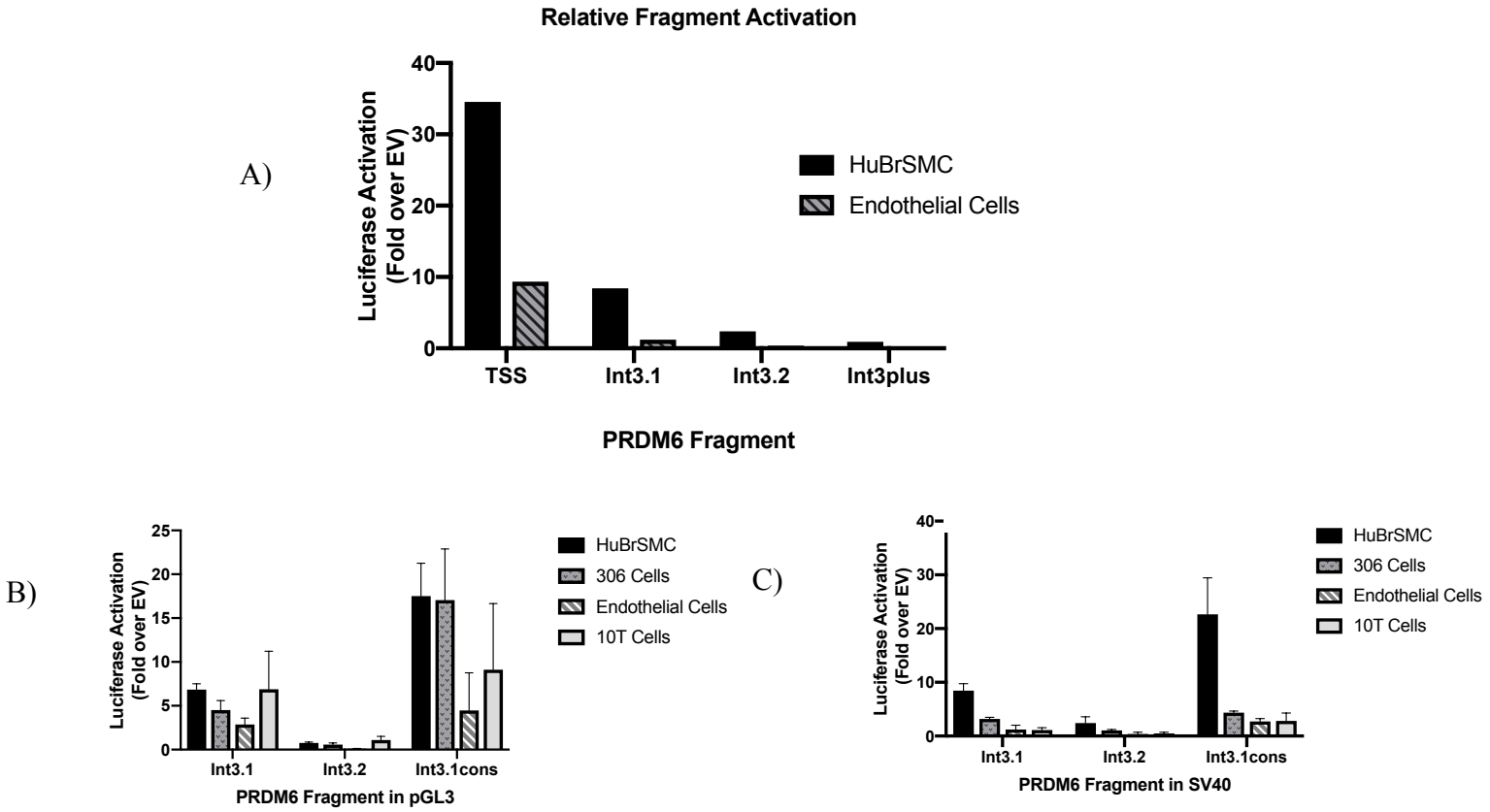


Figure 2. A) PRDM6 TSS and Int3.1 show activation, while Int3.2 and Int3plus show inhibition. We cloned various regulatory regions into Luciferase promoter vectors. Then, we transfected into SMC and non-SMC, then measured Luciferase Activity. **B) PRDM6 has site-dependent regulation.** We cloned PRDM6 fragments into pGL3 vector and transfected into various cell cultures, and measured Luciferase activity to assess promoter-like function.
C) PRDM6 enhancer function is region-selective. We cloned PRDM6 fragments into pGL3-SV40 vector, and transfected into various cell cultures. We then measured Luciferase activity to assess enhancer function of regions.

As shown in Figure 2A, a 2.4Kb fragment containing the PRDM6 TSS exhibited very strong promoter activity in human bronchial (HuBr) SMC (35 fold over empty pGL3 basic vector), an activity on par with several of the traditional SMC marker gene promoters. Importantly, TSS-luciferase activity in endothelial cells was only 9 fold providing evidence that the TSS plays a role in mediating PRDM6's SMC-specific expression.

The Int3.1 fragment containing a PRDM6 SMC-specific DHS displayed strong promoter activity (6.8 fold) when cloned into the pGL3 basic vector (Figure 2B), and even stronger and more SMC-specific activity when cloned upstream of the SV40 promoter (Figure 2C). Interestingly, the Int3.2 fragment exhibited little to no activity in the context of the pGL3 basic

vector but did act as a weak enhancer when cloned upstream of the SV40 promoter. Interestingly, the Int3.2 region inhibited SV40 promoter activity in the non-SMC types strongly suggesting that its repressor function is cell-type specific.

Due to the relative proximity of the Int3.1 and Int3.2 regions, the presence of the eQTLs in this region, and the differential effects of these two regions, a major goal of my project was to test whether the Int3.1 and Int3.2 regions function coordinately. To test this we cloned a 6Kb fragment **(Int3Plus)** and transfected this construct into mouse HuAoSMC and HuBrSMC. Somewhat surprisingly, the Int3Plus region did not drive much luciferase expression in HuBrSMC suggesting that the Int3.2 (or perhaps another Int3pluis region) inhibits the positive transcriptional activity of the Int3.1 fragment.

To test whether human variations affect Int3plus activity, our approach was to make mutations consistent with eQTL variants (Figure 1) in the Int3plus region and assess for changes in transcriptional activity. However, due to the difficulty in cloning the 6Kb Int3plus region we began by testing whether other human variations in the Int 3.2 element affect its activity. In brief, we PCR amplified the Int3.2 fragment from four different human patient samples and from a second cultured cell line of HuSMC, and cloned them into the pGL3 basic and promoter vectors to look for differences in transcriptional activity. We identified several variations in this region by sequencing, but none showed significant effects on the inhibitory activity of the Int3.2 fragment.

Cis-Binding Elements Regulate PRDM6 Activity. Since the Int3.1 domain contains a highly conserved sequence that bound SRF and RBPJ and that contained potential cis elements for these factors (See Figure 1), we hypothesized that this 300 bp region **(Int3.1cons)** mediated the

relatively strong activity of Int3.1 fragment. Of note, we also identified a TEAD (Transcriptional Enhancer Activator Domain) binding site just upstream of the consensus RBPJ cis element. TEAD proteins comprise a family of proteins that bind the MCAT *cis*-acting regulatory element[7]. Specifically, TEAD1 has been shown to negatively correlate to smooth muscle-specific gene expression[8]. Moreover, TEAD1 not only co-regulates SMC genes with SRF [8], but we have shown that it also interacts with RBPJ within the same binding regions to alter SMC differentiation (Mangum et. al; manuscript under review at *Am J Physiol*).

As shown in Figure 2, Int3.1cons had a significantly higher activation across cell types, but most notably in HuBrSMC. Piecing together the high levels of SMC-specific activation, mammalian conservation, and transcription factor binding motifs, we focused our approach on the function and regulation properties of the Int3.1cons region.

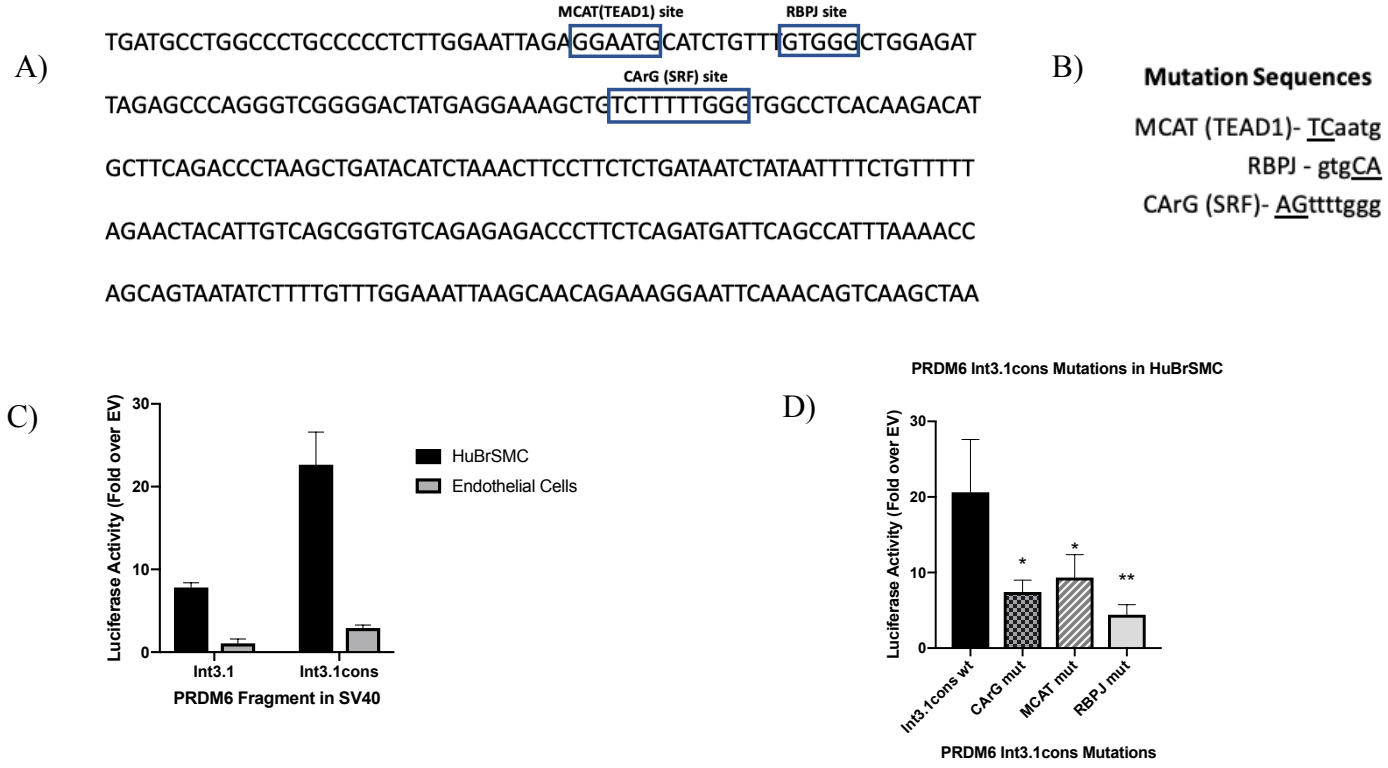
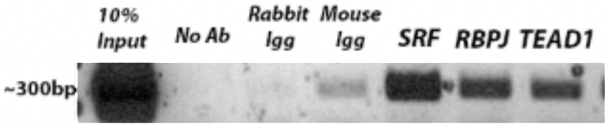


Figure 3. A) PRDM6 Int3.1cons region has conserved cis-binding motifs. Sequence of PRDM6 Int3.1conserved region (red line in Figure 1) with highlighted TEAD1, RBPJ, and SRF binding sites. **B) PRDM6 Int3.1cons mutation sequences.** The identified mutation sequences of Int3.1cons that were cloned into SV40 and transfected to measure change in activity. Mutated base pairs are underlined and capitalized. **C) PRDM6 Int3.1cons has increased activation compared to Int3.1.** We cloned Int3.1 and Int3.1cons into pGL3-SV40 and assessed the luciferase activity in SMC and non-SMC cultures. **D) PRDM6 Int3.1cons mutations significantly decrease activity.** Point mutations were made in Int3.1cons fragment and then cloned into pGL3-SV40 vector. The fragments were then transfected into HuBrSMC, and luciferase activity was measured.

Utilizing a PCR based approach, we created point mutations in the Int3.1cons fragment to test whether these binding sites were important for Int3.1cons activity. Three mutations were created at the SRF binding site, RBPJ binding site, and a suspected TEAD1 (MCAT cis-element) binding site (Figure 3B). After cloning into the SV40 promoter vector, the mutation fragments all had significantly decreased activation from the wild-type fragment in HuBrSMC (Figure 3D). Therefore, the conservation of the wild-type transcription factor binding sites must be pivotal for the normal enhancer function of the Int3.1cons region, suggesting that these binding sites drive the regulation of PRDM6.

Since the Int3.1cons region had transcription factor binding sequences that when mutated, altered activity levels, we used ChIP assays to test whether the region's binding characteristics physically bound to expected transcription factors. ChIP assays use antibodies bound to magnetic beads to pull down DNA-bound proteins in fixed cells. Then, by PCR amplifying DNA primer specific fragments, we can identify which proteins were bound to specific DNA regions. Using HuBrSMC, we discovered that the Int3.1cons region significantly binds to SRF, RBPJ, and TEAD1 when compared to normal IgG antibody IP (Figure 4). Furthermore, we preliminarily observed that SMAD4 and SMAD2/3 (transcription factors activated by TGF-β signaling) were also immunoprecipitated with the Int3.1cons region. The TGF-β signaling pathway is the strongest activator for SMC differentiation, and SMAD2/3 has been shown to bind to SRF to regulate SM α actin[19,20], suggesting that this enhancer may also be regulated by the TGF-β pathway.

A)



B)

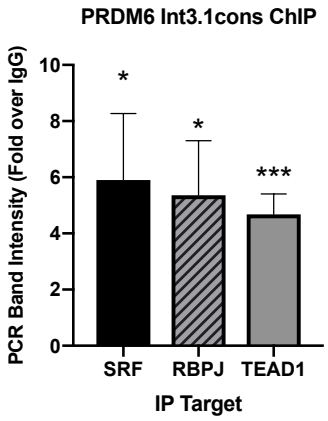


Figure 4. A) PCR band of PRDM6 Int3.1cons shows binding to SRF, RBPJ, and TEAD1. SRF, RBPJ, and TEAD1 were immunoprecipitated and the bound DNA was then eluted and Int3.1cons primers were used to visualize binding levels. **B) PRDM6 Int3.1cons binds significantly to regulatory transcription factors.** Quantification of PCR results from 3 separate ChIP assays shows statistically significant binding compared to normal IgG.

To further test the involvement of RBPJ/Notch signaling on endogenous PRDM6 expression, we measured PRDM6 levels by RT qPCR in HuBrSMC treated with siRNA to RBPJ. Preliminarily results suggest that PRDM6 levels were considerably decreased in RBPJ-depleted cells versus cells treated with an siRNA to GFP (Figure 5).

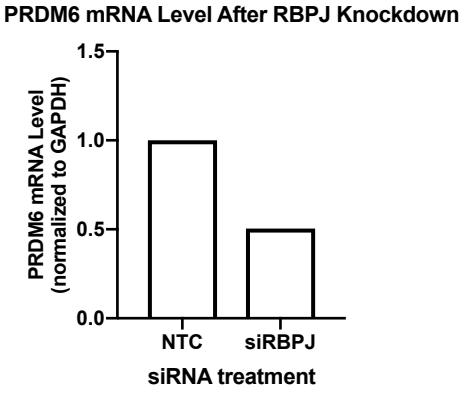


Figure 5. PRDM6 mRNA Levels decrease after RBPJ knockdown. RBPJ was knocked down using siRNA, and PRDM6 mRNA levels were quantified using RT-qPCR.

With the knowledge that specific transcription factors are binding to the enhancer region, we tested what effects the overexpression of the specific signal pathways had on the activity of the Int3.1cons region. In brief, we co-transfected Int3.1cons-luciferase into HuBrSMC with along with FLAG/Myc-tagged expression vectors containing either the SRF co-factor, Myocardin, the RBPJ co-factor NICD, or TEAD1 (the TEAD factor thought to regulate gene

expression in SMC). As shown in Figure 6, we observed that Myocardin overexpression caused a significant increase in activation of the Int3.1cons providing additional evidence that SRF signaling is important for Int3.1cons activity. In contrast, over expression of NICD and TEAD1 inhibited Int3.1cons, although these results were not statistically significant. Importantly, the overexpression of NICD and TEAD1 also decreased the activity of pGL3 basic empty vector suggesting that over-expression of these factors may be down-regulating other aspects of transcription and/or translation or could be causing cellular toxicity.

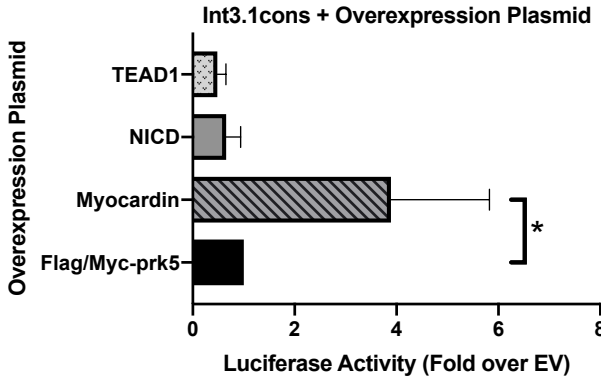


Figure 6. Myocardin overexpression showed significant activation of Int3.1cons. Int3.1cons was cloned into pGL3 vector and transfected with either Myocardin, NICD, or TEAD1 expression plasmids. Luciferase activity was measured and compared to Int3.1cons in pGL3 with Flag EV or Myc-prk5 EV expression plasmids.

Since previous results from the Mack lab suggested that RBPJ and TEAD1 work in unison, we used an siRNA approach to knockdown TEAD1 in HuBrSMC, and subsequently completed a ChIP assay for RBPJ binding levels in comparison to non-targeted control siRNA transfected cells. Interestingly, RBPJ binding to the Int3.1cons region decreased drastically when TEAD1 expression levels were knocked down, suggesting that TEAD1 expression levels are essential for RBPJ binding to Int3.1cons and the regulation of PRDM6 expression in SMC (Figure 7). Importantly, we observed no change in total RBPJ expression levels after TEAD1 knockdown, suggesting that our results were due to changes in binding and not changes in RBPJ protein levels.

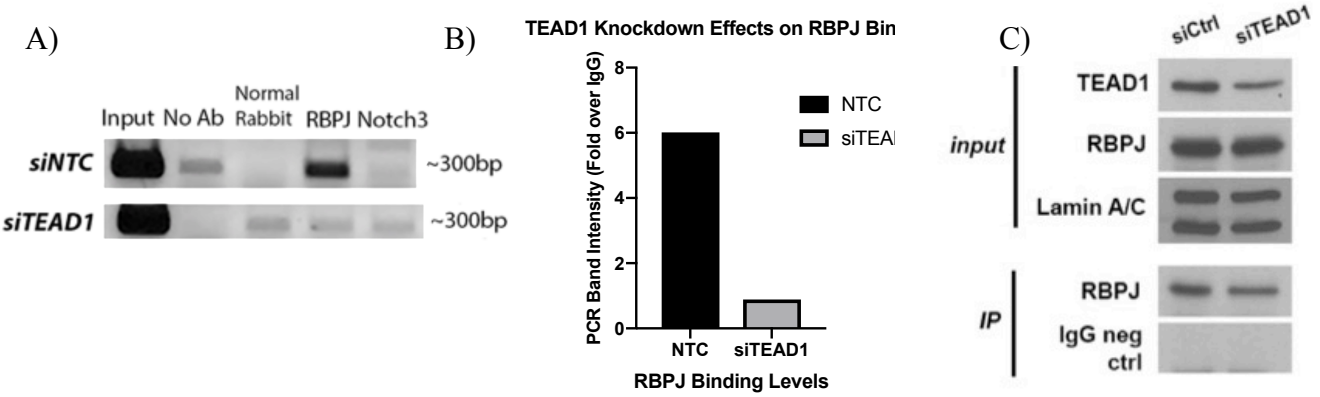


Figure 7. A) PCR bands of PRDM6 Int3.1cons show decrease of RBPJ binding in TEAD1 knockdown. Using an siRNA-mediated approach, we knocked down TEAD1 in HuBrSMC and then conducted a ChIP assay probing for RBPJ and Notch3. **B) Quantification of PCR band shows significant decreases in RBPJ binding when TEAD1 is knocked down.** We quantified the results seen from PCR, however statistical significance cannot be calculated until further experiments are conducted. **C) Western Blot confirms TEAD1 knockdown.** TEAD1 is shown to knockdown when siRNA treated. RBPJ levels do not decrease endogenously from the knockdown of TEAD1. Lamin A/C shows loading control of western blot, and RBPJ levels are consistent from the IP.

The Effects of PRDM6 Expression. To test whether PRDM6 regulated SMC-specific gene expression we used gain and loss of function approaches in SMC cultures. We first cloned the four reported PRDM6 splice variants (see [13] and Figure 8A) into the Flag expression vector and over-expressed them in 10T ½ multipotential cells. These cells can be induced to express SMC markers under various conditions. We ran a Western Blot and then first probed for PRDM6 levels using an anti-Flag Ab. The splice variants 3, 4, and 33 were strongly expressed while variant 36 was expressed at much lower levels suggesting that the loss of the fourth Zinc finger in variant 36 causes the protein to be considerably more unstable (Figures 8B).

We then stripped the membrane and probed for SM marker gene expression using available antibodies to these proteins. We observed little change in SM-marker gene expression, although SM MHC and SM22 were slightly down regulated in the variant 36 over-expressing cells, reiterating the previous literature, which cites that PRDM6 levels affect sm-marker gene expression (Figure 8C). Furthermore, the lower levels of sm-marker genes were most prevalent

in the variant 36, which was the lowest expressed PRDM6 variant, which suggests that a certain level of stable PRDM6 is needed for normal sm-marker expression.

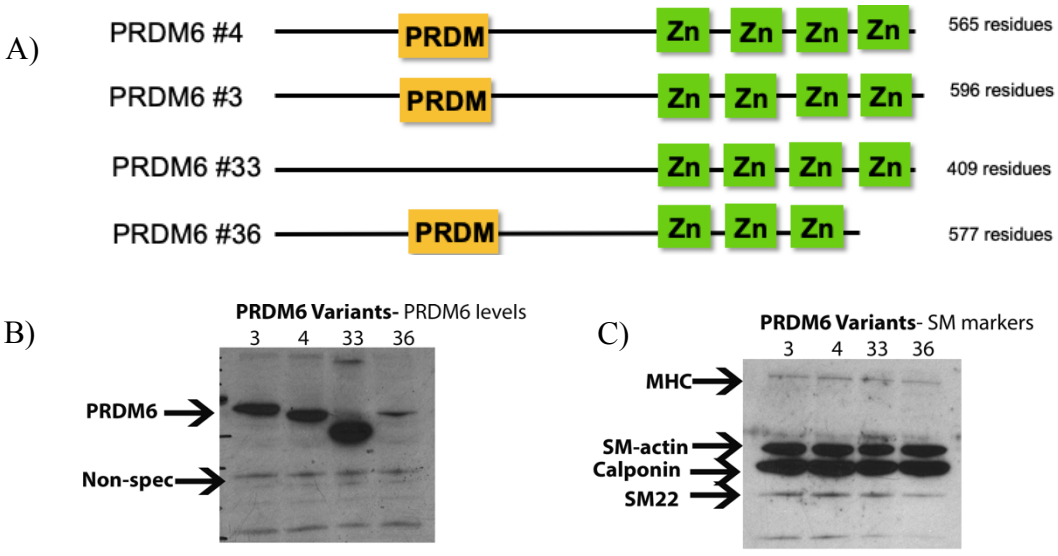


Figure 8. A) PRDM6 splice variant structures. PR/SET= methyltransferase domain. Zn= Zinc finger DNA binding site[13].
B) PRDM6 splice variants were probed using flag antibodies. Constructs were transfected and then Western blotted. **C) SM-marker proteins that were probed using mouse and rabbit antibodies**. We transfected PRDM6 splice variants and then ran a Western blot.

To test whether PRDM6 was required for SM marker gene expression we attempted to deplete PRDM6 expression in HuBrSMC using siRNA. Unfortunately, PRDM6 expression levels in control cells were extremely low and we did not observe significant knockdown of PRDM6 mRNA in these experiments. Nevertheless, we stripped and probed this blot for SM-markers, and not surprisingly, there were no obvious alterations of SM MHC or other marker levels between the control and the PRDM6 knockdown cells (Figure 9).

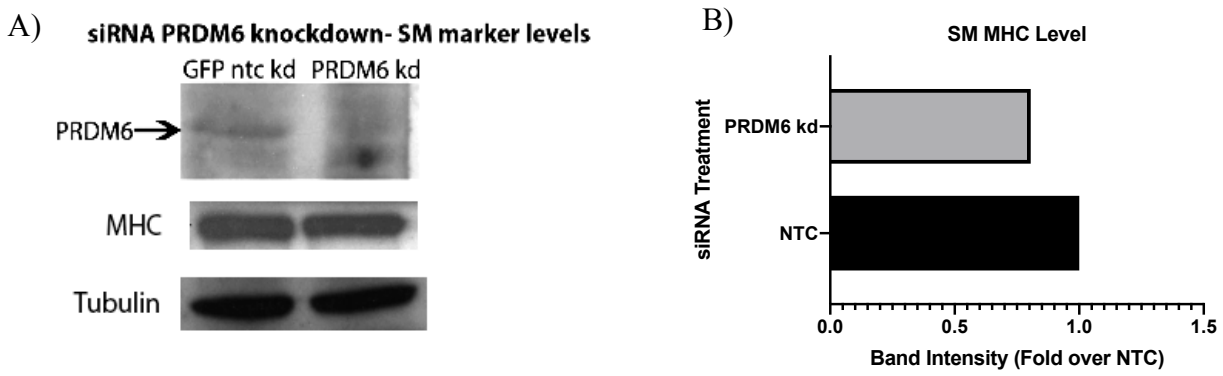


Figure 9. A) PRDM6-targeted siRNA knockdown shows successful knockdown of PRDM6. I transfected siRNA into cells and then probed for PRDM6 expression after running a Western blot, and no bands were found on PRDM6 k/d, but there was a small band in the GFP-targeted control. MHC bands below showed possible change in levels and tubulin was used as a loading control. **B) Quantification of PRDM6-targeted siRNA knockdowns effects on SM markers.** I transfected into cells and then western blot was probed for sm-marker, which showed only slight variation in levels of SM MHC.

Discussion

Regulation of PRDM6 Transcription. We identified that the 2.4Kb TSS promoter region of PRDM6 is highly active in SMC, compared to endothelial cells, which confirms our compiled UCSC data that there is specificity to PRDM6 expression. We also identified that the tested PRDM6 intronic regions were SM-specific in their activity, regardless of whether the fragment activity levels suggested inhibition or activation.

The high levels of activation of the Int3.1cons, the mutagenic approach to Int3.1cons, and the significant ChIP assay binding confirmed our hypothesis that the three known transcription factor binding sites within Int3.1cons play an important role in the regulation of PRDM6 transcription in HuBrSMC. Although, it is still not fully understood in what ways the binding of SRF, RBPJ, and TEAD1 directly control expression, we believe that these transcription factors are binding to the cis elements of Int3.1cons, and either binding directly to the TSS region and driving expression or binding to other transcriptional machinery to control expression of PRDM6 (Figure 10). This interaction could be due to the close proximity in 3-dimensional space that occurs with these regions configured by CTCF and Cohesin proteins that are known to stabilize

theses looping structures of chromatin[21]. However, further experiments must be conducted to test transcription factor interactions, conformational changes and in what ways signaling pathways recruit other regulatory elements.

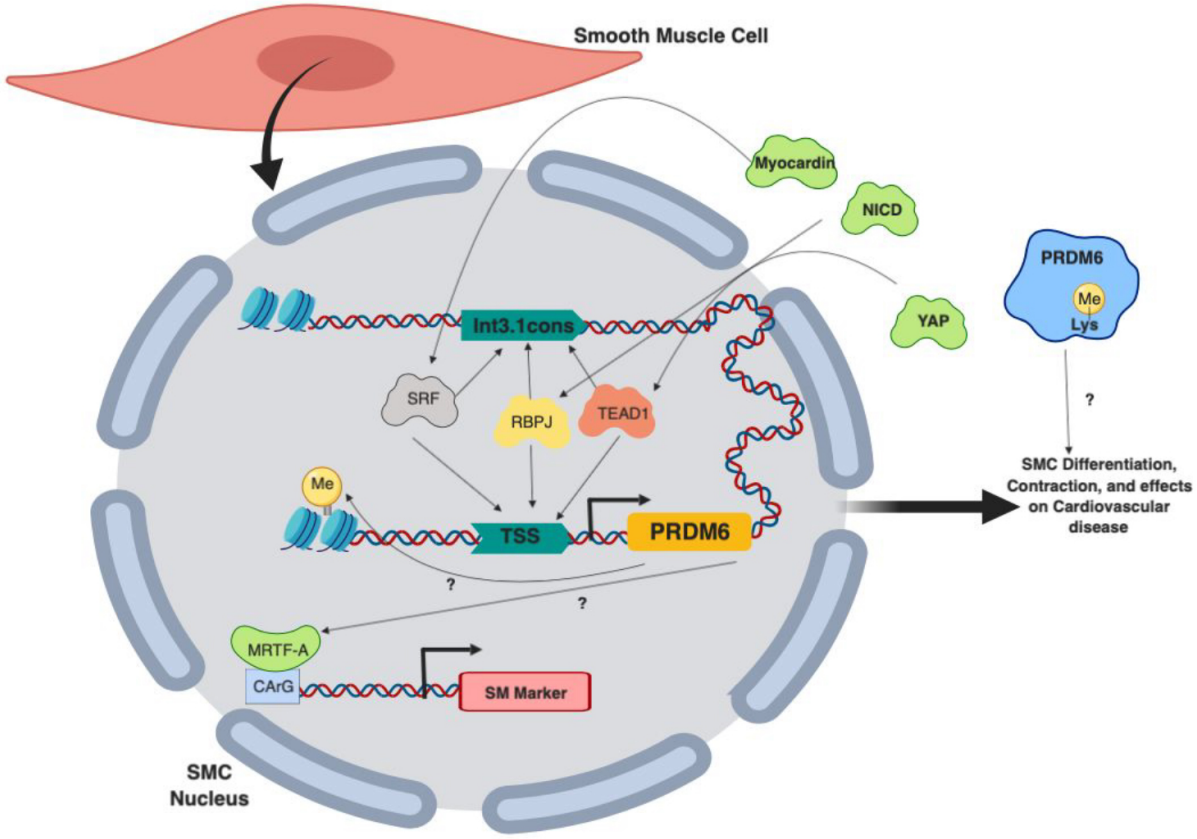


Figure 10. Dynamics of SRF, RBPJ, and TEAD1 regulated PRDM6 transcription in SMC. SRF, RBPJ, and TEAD1 interact with their secondary moieties (Myocardin, NICD, and YAP, respectively) to bind Int3.1cons and drive transcription of PRDM6 and affect SMC function and differentiation.

We failed to identify human variations that alter the Int3.2 inhibition. By combing LD block, GWAS and eQTL data, we will make further combinations of mutations to identify other possible human variations that affect the activity of the Int3plus region. This region is a major focus due to its inhibition of the Int3.1 region when combined into the Int3plus fragment. This could be due to a conformation change in the accessibility of the regions that drive the Int3.1 activity, or other unidentified binding factors that are somehow inhibiting the activating machinery from properly functioning upstream. As seen in Figure 1, the Int3Plus region also

includes identified variations that are shown to correlate to altered levels of PRDM6 expression in hypertensive patients. Finally, with the conditions adjusted to properly clone this fragment, we will follow up by sequencing the Int3plus region and comparing its activity with that of the Int3plus region with point mutations that are consistent with the eQTL and GWAS variations to see if this repressor-like affect is consistent, or if mutations affect the activity of Int3plus.

Upon mutating either the TEAD1, SRF, or RBPJ binding motifs in the Int3.1cons region, we observed a significant decrease in the activity levels of the Int3.1cons fragment, when there was a mutation in either of the three known transcription factor binding sites. This mutagenic approach confirmed our hypothesis that the three known transcription factor binding sites within Int3.1cons play an important role in the regulation of PRDM6 transcription in HuBrSMC.

Furthermore, the ChIP assays in HuBrSMC confirmed that SRF, RBPJ, and TEAD1 are, in fact, binding significantly to the Int3.1cons region. The significant binding of these transcription factors supports our ChIP sequencing data that previously suggested that SRF and RBPJ bound to this region (Figure 1). However, the discovery that TEAD1 also binds to Int3.1cons, opened another method to regulate PRDM6, as well as arising the question of PRDM6 functional involvement in the TGF-β pathway.

We showed that RBPJ binding to Int3.1cons decreased with the absence of TEAD1, suggesting that TEAD1 is necessary for RBPJ binding as well. This finding confirmed our hypothesis that was based around evidence that when RBPJ and TEAD1 are found in close proximity, they co-regulate activity levels (Mangum et. al; manuscript under review at *Am J Physiol.*). Further, our findings imply that TEAD1 may cause conformational changes to the Int3.1cons region to enhance the ability for RBPJ to bind. Moreover, TEAD1 and RBPJ could

interact while bound to the DNA to improve stability, and without TEAD1, RBPJ may not be as stable while bound to Int3.1cons.

Interestingly, we also showed that when Myocardin was overexpressed in HuBrSMC with the transfected Int3.1cons fragment, the activity of the region significantly increased. The significant activation of Int3.1cons, suggests that the activity of Int3.1cons is dependent on Myocardin coactivation with SRF, which has been shown to be essential for inducing SMC differentiation[3,4]. With the SRF region within close proximity of the tightly-knit RBPJ and TEAD1 binding motifs, we hypothesize that these three transcription factors may cooperate and/or compete to regulate transcription levels. Thus the dynamics of SRF, RBPJ and TEAD1 and their corresponding co-factors, Myocardin, NICD and YAP are likely important for PRDM6 expression, and we will be following hypotheses that test their interactions and cooperativity.

When we overexpressed TEAD1, we showed a statistically insignificant decrease in Int3.1cons activity. Although these results are consistent with literature that shows that Hippo pathway/TEAD1 repress SMC differentiation[8], the identification of a significant decrease in activity of the Int3.1cons region when we mutated theTEAD1 binding site (MCAT) is not consistent with either TEAD1 functioning as a repressor in this context. The possibility of other recruited proteins causing the effect on activity levels is a hypothesis that should be explored. By expressing the TEAD1 cofactor, YAP, we may be able to more confidently understand the effects of TEAD1 binding on PRDM6 levels. However, further experiments must be done to understand the downstream effects of TEAD1 binding to Int3.1cons.

Lastly, we preliminarily showed that when RBPJ is knocked down in HuBrSMC, PRDM6 mRNA levels decrease. From this, we can assume that not only is RBPJ significantly binding to intronic regulatory regions, but it also directly affects the PRDM6 expression levels.

Calculating these values are achieved by normalizing to the GAPDH control levels and the GFP-targeted knockdown control. Further replicates will need to be conducted to minimize error bars and determine statistical significance.

PRDM6 Regulation on SM Differentiation. We also showed that none of the PRDM6 splice variants showed a significant effect on SM-markers (Figure 8A). Perhaps other components are required to mediate the effects of PRDM6 on SMC-marker gene expression levels. Low transfection efficiencies would also make it more difficult to detect changes in our model. However, we did preliminarily identify the lower expression level of the variant #36, which may suggest that it is an unstable variant of PRDM6.

The results of my Western analysis for PRDM6 expression indicated that the siRNA protocol knocked down PRDM6 in our human SMC cultures. However, we did not notice any significant change to SMC marker gene expression that has been seen in previous experiments. Further, the lack of mRNA level changes, suggests that our knock down did not actually work. However, these mRNA levels had high error rates and replicates would need to be conducted with improved efficacy. We are presently evaluating the conditions to better understand how to improve the siRNA knockdown of PRDM6. One obstacle is that the endogenous PRDM6 levels are low in our cell cultures, and there is not a strong antibody that allows PRDM6 to easily be identified on Western blots. The low levels of PRDM6 in our SMC cultures suggests that PRDM6 may be important during specific stages of development or differentiation. Moreover, PRDM6 could also play a role in the specific structural integrity of the SMC and its extracellular space *in vivo*, however PRDM6 may not be expressed within cell culture plates that do not experience the stretch or strain of the human vasculature. Further, the mRNA levels of PRDM6

are often too low to amplify in these PRDM6 knockdown samples. Once the appropriate conditions are met, we will be able to further investigate the effects that PRDM6 knockdown has on SMC markers.

Future Directions. We are first interested in identifying what the binding factors of PRDM6 are, to better understand PRDM6 function. In future experiments, we will be conducting further replicates of the transcription factor knockdowns experiment and analyzing PRDM6 mRNA levels. To further understand the dynamics of the binding factors to Int3.1cons, we will also be replicating the siRNA-mediated TEAD1 knockdowns, as well as knockdowns of SRF and RBPJ to analyze how the knockdown of each transcription factor effects the binding of the others to Int3.1cons. Moreover, we will continue overexpression of NICD, myocardin, and YAP with Int3.1cons mutation plasmids. We hypothesize that the mutated fragments will not be as affected by the overexpression. To understand more acutely what drives PRDM6 expression, we will also like to perform knockdowns of SRF, TEAD1, and replicates of the RBPJ knockdown to assess the effects on PRDM6 mRNA levels. We also are interested in conducting ChIP assays on the TSS region and other regions of the upstream PRDM6 introns, with the further goals to understand how transcription factors and signaling pathways interact with PRDM6 regulatory regions to control PRDM6 expression. Importantly, we will be conducting Chromatin Conformation Capture (3C) experiments to test our hypothesis that the intronic regulatory regions are directly interacting with the PRDM6 TSS site.

We would also like to test whether PRDM6 alters histone methylation at the SMC-specific promoters. We would use Chromatin Immunoprecipitation (ChIP) assays in control and PRDM6 knockdown SMC to test the hypothesis that PRDM6 alters H3K4 methylation and/or

other positive histone methylation marks at these genes. We would also test how chromatin modifications affect sm-marker gene expression.

Supplemental

Figure 3. All compared to wt Int3.1cons- CArG mut (unpaired t-test, 3.68, 0.01, 6), MCAT mut (unpaired t-test, 2.96, 0.025, 6), RBPJ mut (unpaired t-test, 4.55, 0.004, 6).
Figure 4. All to normal IgG -SRF (unpaired t-test, 3.59, 0.023, 4), RBPJ (unpaired t-test, 3.90, 0.018, 4) and TEAD1 (unpaired t-test, 8.82, 0.0009, 4)
Figure 5. Flag- Myocardin (ratio paired t-test, 4.60, 0.044, 2)

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References

1. **Mack CP, Owens GK. Regulation of smooth muscle alpha-actin expression in vivo is dependent on CArG elements within the 5' and first intron promoter regions. *Circ Res.* 1999;84(7):852-861.**
2. **Mack CP, Thompson MM, Lawrenz-Smith S, Owens GK. Smooth muscle alpha-actin CArG elements coordinate formation of a smooth muscle cell-selective, serum response factor-containing activation complex. *Circ Res.* 2000;86(2):221-232.**
3. **Wang D, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E, et al. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell.* 2001;105(7):851-862.**
4. **Wang Z, Wang DZ, Pipes GC, Olson EN. Myocardin is a master regulator of smooth muscle gene expression. *Proc Natl Acad Sci U S A.* Jun 10 2003;100(12):7129-7134.**
5. **Rozenberg Julian M, Taylor JM, Mack CP; RBPJ binds to consensus and methylated *cis* elements within phased nucleosomes and controls gene expression in human aortic smooth muscle cells in cooperation with SRF, *Nucleic Acids Research*, Volume 46, Issue 16, 19 September 2018, Pages 8232–8244**
6. **Gridley T. Notch signaling in the vasculature. *Curr Top Dev Biol.* 2010;92:277-309.**
7. **Fu V, Plouffe SW, Guan KL. The Hippo pathway in organ development, homeostasis, and regeneration. *Curr Opin Cell Biol.* Dec 2017;49:99-107.**
8. **Liu F, Wang X, Hu G, Wang Y, Zhou J. The transcription factor TEAD1 represses smooth muscle-specific gene expression by abolishing myocardin function. *J Biol Chem*. 2013;289(6):3308-16.**

9.. Rozenberg JM, Tesfu DB, Musunuri S, Taylor JM, Mack CP. DNA methylation of a GC repressor element in the smooth muscle myosin heavy chain promoter facilitates binding of the Notch-associated transcription factor, RBPJ/CSL1. *Arterioscler Thromb Vasc Biol.* Dec 2014;34(12):2624-2631.

10.. Lockman K, Taylor JM, Mack CP. The histone demethylase, Jmjd1a, interacts with the myocardin factors to regulate SMC differentiation marker gene expression. *Circ Res.* Dec 7 2007;101(12):e115-123.

11. Hohenauer T, Moore AW. The Prdm family: expanding roles in stem cells and development. *Development.* Jul 2012;139(13):2267-2282.

12. Davis CA, Haberland M, Arnold MA, Sutherland LB, McDonald OG, Richardson JA, et al. PRISM/PRDM6, a transcriptional repressor that promotes the proliferative gene program in smooth muscle cells. *Mol Cell Biol.* Apr 2006;26(7):2626-2636.

13. Wu Y, Ferguson JE, 3rd, Wang H, Kelley R, Ren R, McDonough H, et al. PRDM6 is enriched in vascular precursors during development and inhibits endothelial cell proliferation, survival, and differentiation. *J Mol Cell Cardiol.* Jan 2008;44(1):47-58.

14. Li N, Subrahmanyan L, Smith E, Yu X, Zaidi S, Choi M, et al. Mutations in the Histone Modifier PRDM6 Are Associated with Isolated Nonsyndromic Patent Ductus Arteriosus. *Am J Hum Genet.* Oct 6 2016;99(4):1000.

15. Kato N, Loh M, Takeuchi F, Verweij N, Wang X, Zhang W, et al. Trans-ancestry genome-wide association study identifies 12 genetic loci influencing blood pressure and implicates a role for DNA methylation. *Nat Genet.* Nov 2015;47(11):1282-1293.

16. Gaal EI, Salo P, Kristiansson K, Rehnstrom K, Kettunen J, Sarin AP, et al. Intracranial aneurysm risk locus 5q23.2 is associated with elevated systolic blood pressure. *PLoS Genet.* 2012;8(3):e1002563.

17. Li S, Wang DZ, Wang Z, Richardson JA, Olson EN. The serum response factor coactivator myocardin is required for vascular smooth muscle development. Proc Natl Acad Sci U S A. 2003; *100*:9366–93

18. Wang Z, Wang DZ, Pipes GC, Olson EN. Myocardin is a master regulator of smooth muscle gene expression. Proc Natl Acad Sci U S A. 2003; *100*:7129–7134.

19. Qiu P, Feng XH, Li L. Interaction of Smad3 and SRF-associated complex mediates TGF-beta1 signals to regulate SM22 transcription during myofibroblast differentiation. J Mol Cell Cardiol. 2003;35:1407–1420.

20. Mack CP. Signaling mechanisms that regulate smooth muscle cell differentiation. *Arterioscler Thromb Vasc Biol*. 2011;31(7):1495-505.

21. Busslinger, G., Stocsits, R., van der Lelij, P., Axelsson, E., Tedeschi, A., Galjart, N. and Peters, J. Cohesin is positioned in mammalian genomes by transcription, CTCF and Wapl. *Nature. 2017;544,503-507.*